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1 Title Page

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6 Effects of nano-sized zero-valent iron on DDT degradation and residual toxicity in soil: A
7 column experiment

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19 **Abstract**

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Effects of nano-sized zero-valent iron on DDT degradation and residual toxicity in soil:

A column experiment

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Abstract

Background and aims: Nanoscale zero-valent iron (nZVI) application is a promising technology for degradation of chlorinated contaminants in soil. Plants also play an important role in soil remediation and nZVI should not adversely affect plants growing on treated soils. Large amounts of DDT are still found in certain soils and means to remediate these soils are limited. Our aims were to investigate the effect of nZVI on DDT degradation and evaluate possible negative effects of nZVI on plants.

Methods: Columns with spiked (20 mg DDT kg⁻¹) soil was percolated with nZVI (1 g nZVI L⁻¹) and leached with five pore volumes of water to assess leaching of nZVI and residual toxicity of leachates and soil to plants using seed germination and plant growth tests (barley, flax).

Results: Addition of nZVI led to degradation of 45 % of the added DDT. Percolation with water significantly oxidized and transported iron through the columns. The first leachates had negative effects on plant development, but after leaching with 4 pore volumes, neither soil nor leachates affected plant negatively.

Conclusions: nZVI is efficient for degradation of DDT and adverse effects of nZVI on plants seem ephemeral and are alleviated after oxidation mediated by percolating water.

Key words: Chlorinated organics, DDT, Ecotoxicity, nanoparticles, pesticides, polluted soil, remediation.

Introduction

Chlorinated organic pollutants are among the most persistent and toxic contaminants in soil, and pose serious risks to human health and the environment throughout the world. Among these, organochlorine pesticides like DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane], used massively worldwide for three decades after World War 2 to control agricultural pests and malaria bearing mosquitos, are well known (Li et al. 2010; Wong et al. 2005). DDT was subject to an international ban in 1972, but is still used in smaller amounts under strict regulations, even in Europe. One example is Kelthane (Dicofol) (containing 14% DDT isomers) which is used to control acaridae pests in agriculture, and which currently contributes to environmental contamination (Yang et al. 2008). Due to its persistence, DDT residues and its metabolites are thus widely distributed and can be found at polluted sites all over the world (Hitch and Day 1992), and is frequently detected in air, water, soil, sediments, fish, birds and humans. DDT has received a great environmental concern because of its persistence, bioaccumulation and biomagnification in food chains, and its potential toxicity to humans and wildlife (Daly et al. 2007; Eggen and Majcherczyk 2006; Guo et al. 2009; Hinck et al. 2009; Li et al. 2010; Yang et al. 2008).

During the past two decades, several methods have been developed for degradation of DDT, including bioremediation treatments (Li et al. 2010), soil excavation and incineration or thermal degradation at high temperatures (Rodante et al. 1992), photocatalytic techniques using photochemical reactions with TiO₂/UV (Lin and Lin 2007), washing soil with surfactants (Smith et al. 2004) and metal-catalyzed reactions (Pd/C catalysts) (Zinovyev et al. 2005). Bulk sized zero-valent iron has been used for DDT degradation in water and soil with some success (Eggen and Majcherczyk 2006; Sayles et al. 1997; Yang et al. 2010).

Recently, a new technology using nano-sized zero-valent metals for remediation has been developed, being particularly promising for chlorinated organics when employing nanosized zero valent iron (nZVI). The advantages of using nZVI for treatment of contaminated water and soil include: 1) Ability to treat contaminants *in situ*, avoiding costly transportation of soil to remote treatments sites or waste disposals (Karn et al. 2009; Otto et al. 2008). 2) On site, contaminated groundwater need not be pumped out for above-ground treatment (as in “pump and treat”-remediation). 3) Due to their small size, high surface area and special surface coatings, nanoparticles may penetrate and move even within very small soil pores. They may also remain suspended in groundwater for a sufficiently long time to interact with pollutants. Nanoparticles can thus travel farther than larger, macro-sized particles, which facilitates distribution within a contaminated matrix and reduce work and costs in connection with injections. Further, nanoparticle suspensions can be injected from the surface to any location and depth (e.g. underneath buildings). However, nZVI could be less efficient for degradation of contaminants in water and soil compared with larger sized ZVI due to high reactivity and short lifetime (Comba et al. 2011). Several methods do however exist to modify nZVI reactivity, lifetime and mobility. Coating with surfactants, such as polyacrylic acid (PAA) or caboxymethyl cellulose (CMC), has been proven useful in this respect (He et al. 2010; Schrick et al. 2004). Another modification involves incorporation of noble metals like palladium (Pd) and nickel (Ni) that enhance the catalytic properties of nZVI. However, the high cost and environmental concern for spreading heavy metals has limited a widespread use of such bimetallic nZVIs in field applications (Comba et al. 2011; Jiang et al. 2011; Mueller et al. 2012). Comba et al. (2011) and Li et al. (2010) also found that there were no significant difference between mono and bimetallic nZVI for efficient degradation of DDT and other contaminants in soil and water. Still, several studies have shown that bimetallic nZVI is efficient in dechlorination of many chlorinated compound such as trichloroethylene (TCE),

pentachlorophenol (PCP), carbon tetrachloride (CCl₄) (Elliott and Zhang 2001; He et al. 2010; Lien and Zhang 2007; Xu and Zhang 2000; Zhang et al. 1998). Field applications of both types have also been conducted with good results on degradation of compounds like PCB, PCE, TCE, DCE and VC (Karn et al. 2009; result presentations on www.nanoiron.cz and www.nanotechproject.org).

Although this technology may be efficient in degrading chlorinated pollutants in soil, it is also important that such remediation preserves or restores soil quality to permit reuse of soil for a wide range of purposes. The lack of knowledge about possible negative effects of nZVI on plants and soil organisms following its application to soil is therefore an aspect that currently hampers a wider use and large scale implementation of nZVI technology. Toxic effects on plants have been shown during exposure both in the presence and absence of soil (El-Temsah and Joner 2012b; Phenrat et al. 2009). These authors also suggested that oxidation and aging could reduce the adverse effects of nZVI related to the induction of unfavorable red-ox conditions. Leaching of water through treated soil may move nZVI away from an injection point and lead to dilution. Also, the oxygen contained in leaching water may oxidize nZVI and raise Eh to a level where O₂ availability to aerobic organisms is no longer critical. To our knowledge, these aspects have not been examined in an ecotoxicological context. The objectives of the present work were thus; 1) to investigate the effect of monometallic nZVI coated with CMC on the degradation of DDT in soil columns, 2) to assess the impact of leaching water on movement of nZVI and other Fe species, and 3) to measure possible negative effects on plants of nZVI in leaching water and leached soil. The possible contribution of boron and Fe²⁺ to the observed toxic effects was also examined.

Materials and methods

Synthesis of nano-sized zero valent iron

Nano-particles of zero-valent iron stabilized with carboxymethyl cellulose (CMC) was prepared by the borohydride method with ferrous ion, as described by He et al. (He et al. 2010), without using Pd. Briefly, 5 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 450 mL water immediately before use and mixed with 5 g CMC in 450 mL water. The mixture was then shaken for about 15 min to ensure formation of Fe^{2+} -CMC complexes. ZVI nanoparticles were then formed by reducing Fe^{2+} ions using a borohydride solution (30 mL of a 1.9 M, introduced at 5 mL min^{-1}). The resulting suspension was adjusted to 1 L and contained 1 g Fe L^{-1} . The suspension was shaken until hydrogen evolution ceased to ensure efficient use of BH_4^- . The size of the resulting nZVI particles, measured using high resolution transmission electron microscopy (JEM-2011; Jeol, Japan, operating at 200 keV), was in the range 20-100 nm. The hydrodynamic diameter and zeta potential, measured by dynamic light scattering (DLS) and phase analysis light scattering (PALS), respectively, using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., England) showed particle size between 178 and 424 nm and a zeta potential of -42.8 mV (previously described in El-Temsah and Joner, 2012b).

Column experiment

Triplicate PVC tubes (40 cm long, 2.5 cm diam.), cut longitudinally and joined with silicon glue to facilitate separation at harvest, were filled with 250 g d.wt. sandy loam soil (85% sand, 11% silt, 4% clay, 1.1% organic matter, pH 5.8, sieved $< 2 \text{ mm}$). One day before filling the columns and starting the nZVI treatment, the upper 50 g of soil in each column were amended with $20 \text{ mg DDT kg}^{-1}$ (PS-74, Chem Service Inc., West Chester, PA, USA; containing 18 % o,p' DDT and 77 % p,p' DDT). DDT was dissolved in hexane (1 mg mL^{-1}) and added to 10 % of the soil volume (dried soil), evaporated over-night and mixed with humid soil (90 % on a dry weight basis). This soil was placed on top of each column, and separated from the soil below with disks of medical cotton cloth to facilitate the separation of spiked and non-spiked soil at harvest. Columns were saturated with deionized water, left to equilibrate for 6 h and then

received 50 mL of a freshly made and continuously agitated nZVI suspension (described above) added drop-wise from the top with a pipette. Triplicate columns without nZVI were also prepared as controls. During the next 5 days, and after leaving the nZVI to react with the DDT-spiked soil at room temperature for 24h, 48h, 72h etc., 50 mL per day of de-ionized water was added to the top of the columns at 2 mL min⁻¹ and leaching water collected in vials placed below the columns. Five days after adding nZVI, the columns were split longitudinally and the soil divided into three sections (the top 50 g of spiked soil and upper and lower half of the underlying soil). These portions of soil were homogenized by mixing and 3 g of soil from each section were taken for DDT analysis and 1 g used for measurements of Fe⁺² and Fe⁺³. The remaining soil from each section was used for seed germination tests.

Seed germination tests

Seed germination was used to test whether leached water or soil could have adverse effects on plants. Soil from each section and leached water from all samplings were used in seed germination tests, and compared to non-treated controls, as described in El-Temsah and Joner (2012). Briefly, ten seeds of barley or flax, representative of monocots and dicots, respectively and previously verified as dose-response sensitive to nZVI, were placed either in the sampled soil at field capacity (in triplicate petri dishes), or on Whatman no. 5 filter paper (in triplicate petri-dishes) amended with 6 mL freshly leached water. Seeds were incubated at 25 °C in the dark (seeds in soil were moved to a growth chamber with 16h/8h light-dark cycle after 24h). Percent germinating and length of roots and shoots were recorded after 5 days in soil or 4 days on filter paper (OECD 2006).

To evaluate which component of nZVI leachates that may cause toxicity, we separated a freshly made nZVI suspension into a particulate fraction and an aqueous fraction by centrifugation (9433 × g, 15 min). Serial dilutions from 0 to 100 % of the supernatant were used in seed germination tests with two species × ten seeds × three replicates, as above: Five mL of

the supernatant was added to 50 g untreated sandy loam soil in 6 cm polypropylene pots, or 6 mL supernatant was added to petri dishes lined with Whatman no. 5 filter paper, and germination percentage, and root and shoot length recorded as above. The effects of boron (as boric acid) and Fe^{2+} (as FeSO_4) on seed germination were also tested using this scheme to establish thresholds for no observed effect concentrations (NOEC) for these components individually.

DDT extraction

Soil samples were analyzed for DDT after extracting 3 g of air dry soil with 10 mL of cyclohexane and 10 mL acetone in glass flasks at 150 rpm on a horizontal shaker (adapted from Tian et al. 2009). After 1 h, 15 mL of deionized water were added and the resulting emulsion shaken for another 5 min. The emulsion was centrifuged at $671 \times g$ for 5 min for phase separation. The cyclohexane phase was then sampled for analysis on GC-MS (GC 6890N and MS 5973N, Agilent, USA) using a $0.2 \text{ mm} \times 50 \text{ m}$ ($0.25 \mu\text{m}$ film thickness) Varian CP7482 capillary column and 1 mL min^{-1} He as carrier gas. A $2 \mu\text{L}$ sample was injected into a split/split less injector (Agilent) at an initial temperature of oven: 80°C , injector: 250°C and column: 325°C . DDT, DDD and DDE were separated by retention times and selective ion mass. The recovery of total DDT from soil was $93.6 \pm 4.8 \%$.

Fe extraction from soil:

Fe^{2+} was measured in fresh leachates using the ortho-phenathroline method (Christian 2004). Fe^{2+} and Fe^{3+} was measured in soil using HCl extraction and a ferrozine reagent (Lovley and Phillips 1986). Approx. 0.5 g of soil was transferred to 5 mL of 0.5 M HCl in a glass vial. The soil and acid were mixed by gentle swirling for 30 s and left for 1 h at room temperature, after which a 0.1 mL sample was extract and added to 5 mL of ferrozine (1 g L^{-1}) in 50 mM HEPES (N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffered to pH 7 using NaOH. The amount of Fe(II) was determined spectrophotometrically by measuring the absorbance of

the supernatant at 562 nm. Fe(II) is not oxidized and Fe(III) is not reduced during such extraction. Another sample of the soil was extracted by the same procedure as above with the exception that the extractant was 5 mL of 0.25 M hydroxylamine hydrochloride in 0.25 M HCl. Under acidic conditions, hydroxylamine reduces Fe(III) to Fe(II). The amount of hydroxylamine-reducible Fe(III) was calculated as the difference between the Fe(II) measured in the hydroxylamine and HCl extractions (Lovley and Phillips 1986).

Boron measurement in water

The principle of the spectrophotometric method for determination of boron is its reaction with azomethine-H, which is the product of 8-aminonaphthyl-1-ol-3,6-pyrosulfuric acid and salicylic aldehyde. In the presence of dissolved forms of borates, at pH=6, formation of a yellow complex takes place, which can be measured spectrophotometrically as described by Edwards (1980). Briefly, 1 mL sample is mixed thoroughly with 2 mL of a buffer-masking solution and mixed with 2 mL of azomethine-H solution. After 30 min, absorbance is measured at 420 nm.

Statistical analysis

For the statistical analysis, a one way analysis of variance (ANOVA) was used to assess the differences in toxic effects between nZVI treatments and controls. Student T-tests were used for comparing differences between means. Probit regression analysis (EPA Probit analysis, v. 1.5, US EPA) was used to determine EC50 and LC50 values (50 % effect concentration or lethal concentration, respectively) using % plant growth inhibition at the different exposure concentrations.

Results

DDT degradation

Addition of nZVI and subsequent leaching with water led to a reduction in DDT concentrations in soil of almost 50 % compared to controls without nZVI (Table 1). DDT in leachates were below the detection limit ($<0.01 \text{ mg L}^{-1}$; data not shown). DDT distribution within the different sections of the soil columns showed low mobility of DDT and limited transport of the metabolites DDE [1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene], and DDD [1,1-dichloro-2,2-bis(p-chlorophenyl)ethane]. Compared to controls, the reduction in DDT concentration in the treated soil was 44.8 %, while extractable concentrations of DDT from the control treatment was 19 % lower than the initial nominal concentration, presumably due to loss by adsorption to pipettes during spiking and to the PVC columns during the experiment. DDT degradation was followed by significant increase of DDD and DDE in soil treated with nZVI. These metabolites were also recovered in higher amounts in the soil below the spiked/nZVI treated soil compared to the soil below spiked/non-treated soil. The recovered metabolites of DDT (DDD and DDE) constituted 1.3 % of the initial concentration of DDT in nZVI-treated soil compared to 0.4 % of the initial DDT concentration in the control soil.

Concentrations of Fe^{2+} and Fe^{3+} in the different soil sections after five leaching episodes are shown in Table 2. Fe^{2+} concentrations in soil significantly increased after nZVI treatment and leaching, while there were only small differences in Fe^{3+} concentrations between soil amended with nZVI and controls, and between spiked soil and the sections underlying it. Concentrations of Fe^{2+} measured in leachates from the soil columns during the 5 days are presented in Table 3. There was a small difference between Fe^{2+} in leachates from control and nZVI treated soil, and there was only an increase in Fe^{2+} from 18 to 25 mg L^{-1} from the control to the highest value recorded (which was found for the 2nd and 3rd leaching event).

Soil-less germination tests

The effects of water leachates on germination of barley and flax in the absence of soil are shown in Table 3. While leachates from control soil permitted a high germination rate, the

first leachate from nZVI-treated columns reduced germination of barely from 93 to 67 % and only reached a germination rate not significantly different from controls after the 3rd leaching. For flax, 100% germination was observed for controls and the first leachate, and only a slight reduction to 93 % for leachates from the 2nd and 3rd day of leaching, after which germination rates increased to 100 % again.

Inhibition of shoot and root development in barely and flax seedlings responded differently to leachates with higher relative inhibition of root growth than for shoot growth (Figure 1). Also, shoot and root growth was a more sensitive indicator of the negative effects of water leached from the soil column than germination percentage. While water from the first leaching had only a modest effect on seedling growth, water from the second and third leaching had severe negative effects on both root and shoot development. Water from the 4th leaching had only weakly negative effects on development of barley and no significant ($p<0.05$) effects on flax, while the 5th leaching had no adverse effects on either plant species.

Seed germinated in soil

The effects of nZVI remaining in soil on germination of barley and flax are shown in Figure 2. As for germination on filter paper, root development was affected to a higher extent than shoot development. Strong negative effects of nZVI addition and leaching were observed in soil from all sections of the soil column with respect to root development of both species. The strongest negative effects were observed in the top layer also containing DDT. Less negative effects on root development were observed for the bottom section of the soil column compared to the soil section closer to the point of nZVI introduction. Shoot development of germinating barley was unaffected for all soil sections, and only moderately affected for flax. Attempts to germinate seeds in soil freshly amended with nZVI (with no leaching) resulted in complete inhibition for both plant species.

Adverse effects of nZVI suspension components

The inhibitory effects of the aqueous phase of the nZVI suspension were evident for both barley and flax when germinated both on filter paper and in soil. The undiluted nZVI aqueous phase caused an approx. 90% reduction in germination on filter paper for both species (Figure 3a). An approx. 50 % reduction was observed when the aqueous phase was diluted to 25 % of its original concentration for barley and to 12.5 % for flax. Shoot development was far less sensitive, but showed the same general trend (results not shown).

When the aqueous fraction of the nZVI suspension was used for seed germination in soil, inhibition was less evident than when germinated on filter paper (Figure 3b). The undiluted aqueous fraction reduced root development in both barley and flax by approx. 50 %, and no inhibitory effects were seen when the aqueous phase was diluted beyond 50 %. Inhibition of shoot development was less pronounced, but followed the inhibition pattern seen for roots (results not shown).

Toxicity of boron and Fe(II)

Seed germination and root and shoot development were negatively affected when B or Fe^{2+} was added to soil, and the dilution series tests permitted us to establish EC50-values for both ions for comparisons with effects from the aqueous phase of the nZVI suspension (Table 4). For B, EC50 values were similar for root inhibition of barley and flax: 13 and 12 mg B kg soil⁻¹, respectively. For Fe^{2+} , EC50 for root inhibition differed strongly between the two species, being 140 mg B kg soil⁻¹ for barley and 40 mg B kg soil⁻¹ for flax. The concentrations of B and Fe^{2+} in the undiluted aqueous phase of the nZVI suspension were 22 and 121 mg L⁻¹, respectively, whereas the Fe^{2+} concentration in the leachates was between 20 and 25 mg L⁻¹, marginally higher than in the control (18 mg L⁻¹).

Discussion

The present study shows that nZVI has a potential for degradation of DDT in surface soil when added in relatively low doses. Effective, inexpensive, rapid and simple methods have been sought for decades to allow remediation and restoration of soils contaminated with recalcitrant chlorinated compounds (Shea et al. 2004; Yang et al. 2010), and nZVI may represent a step-change in remediation this respect. In our study we used 1 g nZVI L⁻¹ for treating spiked soil, which is considered a low concentration for use in field applications. The concentrations in field application might be higher due to environmental and soil conditions such as temperature, soil types and structure. Saleh et al. (2007) suggested that field scale application should employ at least 3 g nZVI L⁻¹, and nZVI slurry concentrations used so far for field applications have more commonly varied between 10 to 50 g nZVI L⁻¹ (Grieger et al. 2010; Phenrat et al. 2009). Increasing doses will however not automatically lead to increased degradation in terms of lower residual concentrations remaining in treated soil, as other factor may become limiting for degradation.

Bulk zero-valent iron has been used previously as a reducing agent that mediate degradation of organochlorine compounds such as DDT, lindane, metachlor, alachlor, chloropyrifos and atrazine in water, soils and/or sediments, and even aged DDT (Boussahel et al. 2007; Eggen and Majcherczyk 2006; Kim et al. 2010; Sayles et al. 1997; Shea et al. 2004) e.g. reaching degradation rates of four pesticides (metachlor, alachlor, chloropyrifos and atrazine) of 60 % after incubation for 90 days with 50 g kg⁻¹ ZVI in soil (Shea et al. 2004). Similary, adding 50 g kg⁻¹ ZVI and 30 % moisture resulted in 91 % and 98 % degradation of metachlor, which has a low solubility (log Kow 3.2) and only one Cl atom, in soil after 3 and 40 days incubation, respectively (Kim et al. 2010). Furthermore, 65 and 93 % degradation of DDT in an aged sediment after incubation with ZVI for 10 and 40 weeks, respectively, has been observed (Eggen and Majcherczyk 2006). Nanosized ZVI has later proven even more efficient for dechlorination of pesticides including atrazine for which 64 % degradation was observed

after 2 h incubation with 2 g L⁻¹ organobentonite nZVI in water (Zhang et al. 2011). Satapanajaru et al. (2008) observed a degradation rate of atrazine in water and soil that was seven times higher when nZVI (20 g L⁻¹) was used compared to ZVI (50 g L⁻¹) in water, while 100 g kg⁻¹ of both nZVI and ZVI was used in soil treatment. Nanosized ZVI was also efficient for DDT degradation in water, with 85 % of DDT degraded in water with nZVI at a concentration of 50 g L⁻¹ after 8h incubation, and there was no significant differences between nZVI and nickel-doped nZVI (Ni-nZVI) (Tian et al. 2009). The differences observed between degradation capacity of ZVI and nZVI is due to the fact that nZVI has a larger surface area and more reactive sites, and therefore a higher efficiency in dechlorination of most chlorinated compounds compared to micro-scale zero-valent iron (Wang and Zhang 1997) (Liu et al. 2005; Zhang et al. 2011).

Oxidation of nZVI is the main parameter affecting nZVI reactivity and toxicity. Infiltrating water from the soil surface, as in this experiment and under field conditions during precipitation, is one source of oxygen driving this process leading to reduced concentrations of Fe⁰ and temporary increased Fe²⁺ concentrations in soil, seen as a spatial peak in Fe²⁺ in the middle section of the nZVI-treated columns: The upper section having received nZVI and subsequently water with O₂ for 5 days contained less Fe²⁺ and more Fe³⁺ than the underlying section. In the bottom section concentrations of Fe²⁺ and Fe³⁺ were comparable to the soil at the top of the column, perhaps due to O₂ diffusion into the soil from the column outlet. According to Satapanajaru et al. (2003), presence of Fe²⁺ and Fe³⁺ during nZVI oxidation is enhancing the dechlorination of metachlor. It is known that the dechlorination occurs when the chlorine moiety accept electrons released during oxidation of nZVI to Fe²⁺ and Fe³⁺. Normally, dechlorination produces more biodegradable metabolites, as indicated by temporal increases in the DDT metabolites (DDD and DDE) in soil after incubation with nZVI. There are two common reductive processes degrading DDT: Dechlorination producing DDD and

dehydrochlorination producing DDE (Quensen et al. 1998). DDT and its metabolites have very low solubility in water. DDT, DDD and DDE water solubility is 3.1-3.4 $\mu\text{g L}^{-1}$, 160 $\mu\text{g L}^{-1}$ and 40 $\mu\text{g L}^{-1}$, respectively (Royal Society of Chemistry 1996). The amounts of DDT transported down through the column ($>20 \mu\text{g}$) are far higher than what can be accounted for by DDT solubilized in percolating water ($<1 \mu\text{g}$). It is therefore likely that some DDT has been adsorbed onto nZVI and transported further down the column on these particles. These amounts still represent only approx. 0.1 % of the initial DDT added to the system, and DDD+DDE even less, and therefore should not represent any danger for enhanced mobility and transport to uncontaminated soil or aquifers.

Effects of nZVI on plants

We have previously shown that nZVI can affect seed germination and plant growth negatively at concentrations below those commonly used in field treatments (El-Temsah and Joner 2012). The present study shows that ecotoxicity tests with plants are also suited for testing potential negative effects in water leaching through nZVI treated soil. Further, we have also shown that oxidation during ageing of nZVI in non-saturated soil partially alleviate this toxicity (El-Temsah and Joner 2012b). These findings are in agreement with those of El-Temsah and Joner (2012a) and Phenrat et al. (2009) who observed that oxidization rendered nZVI non-toxic in cyto- and neurotoxicological tests. Further, partial oxidation of nZVI was shown to reduce the toxic effects on bacteria (*Escherichia coli*) (Li et al. 2010). Changes in a microbial community caused by nZVI could even be reversed after the complete oxidation of nZVI (ageing for 250 d) (Kirschling et al. 2010). In this case, restoration occurred within a long time-span, whereas our experiment showed that a certain functional restoration can be achieved within a far shorter time if oxidation is enhanced e.g. by leaching water.

In our study we tested the effects of two secondary components of nZVI in an attempt to reveal if either of them was causing the observed effects on plant development. Apparently,

the contribution of Fe^{2+} to the observed phytotoxicity of nZVI treated soil or its leachates was low. Even though Fe^{2+} concentrations in soil were 300-450 mg kg^{-1} higher in nZVI-treated soil and underlying soil at the end of the experiment, compared to controls, the reduced growth of seedlings (Fig 2) did not reflect the measured Fe^{2+} concentrations (Table 2). Neither was seed germination of flax (the most Fe^{2+} -sensitive plant we tested) affected to any higher extent than the more Fe^{2+} -tolerant plant, barley, in germination tests on treated soil (Fig 2). On the other hand, the residual boron may contribute to the phytotoxicity of nZVI, as it had EC50 values (12-13 mg B kg^{-1}) that were well below that of the B concentration in nZVI suspensions (22 mg B kg^{-1}) and 4-10 times lower than the EC50 values for Fe^{2+} . However, B is easily leached out of coarse textured soils (e.g. Mertens et al. 2011 and references therein). To avoid negative effects of B altogether, it would be relatively easy to remove excess B by washing nZVI prior to application. This would remove both residual BH_4 and its oxidation product (boric acid). Using washed nZVI or nZVI produced by other methods will thus not cause this type of negative secondary problems and may be preferable in situations where enhanced levels of B are undesirable. Boron is fairly mobile in soil, but has a far lower bioavailability than in water (Butterwick et al. 1989). In the present experiment this led to both elution of added B during leaching and a lower toxicity response when comparing toxicity towards germinating seeds in soil with seed germination on filter paper. The former showed no effect of B, even for the most sensitive plant species used (barley), even though root development was affected at lower concentrations. Our EC-values are in agreement with those of Mertens et al. (2011) who tested boron toxicity on barley in different soils and found EC10 for added B in the range of 3-27 mg kg^{-1} .

The use of nZVI for degradation of chlorinated organics is designed for treating contaminants in ground water and anaerobic subsoil. In surface soils, the presence of oxygen and organic matter will compete with chlorinated substances and react with Fe^0 as to render

dechlorination less effective. In this way, treating surface soils may be less efficient, but if oxygen levels are reduced by prior saturation with water, plus a certain incubation time to allow microbial consumption of dissolved O₂, the efficiency may still be sufficiently high to obtain a significant reduction of the targeted pollutants. The lack of alternative sustainable methods to treat chlorinated organics in nan-saturated soils makes further testing of the nZVI technology important. Our own studies on nZVI-induced DDT degradation in soil polluted in the 1960-ies indicate that even aged DDT may be attained (El-Temsah and Joner, unpublished results). Future experiments should focus on the feasibility to treat such soils and continue to include tests on possible negative effects on plants and soil biota as they are likely to be exposed during and after treatment of surface soils. To the extent that boron from nZVI synthesis using BH₄ creates negative side effects, washing of crude nZVI suspensions or different synthesis methods should be considered.

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Figure captions

Fig 1. Effects of nZVI in water (control), a freshly prepared nZVI suspension at 1 g L⁻¹, and from 5 consecutive leaching episodes of nZVI amended soil columns on (a) root and (b) shoot length of barley and flax germinated on filter paper. Means for the same plant species associated with the same letter are not significantly different (Student-t test, $p < 0.05$, $n = 3$)

546

547 **Fig 2.** Root and shoot length of (a) barley and (b) flax germinated in unamended soil
548 (control), soil receiving freshly prepared nZVI at 1 g L⁻¹, and in soil from columns treated
549 with nZVI after five leaching episodes. Within roots or shoots, means associated with the
550 same letter are not significantly different (Student-t test, $p < 0.05$, $n = 3$)

551

552 **Fig 3** Effects of the aqueous phase of nZVI (100 % supernatant fraction = 1 g L⁻¹, and five 2-
553 fold dilutions) on seed germination (percentage noted for individual bars) and root
554 development of barley and flax germinated on filter paper (a) and in soil (b). Within species,
555 means associated with the same letter are not significantly different (Student-t test, $p < 0.05$,
556 $n = 3$)